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Correlation Between the Intravenous Administration of Methemoglobin Formers and *in vitro* Monitoring of Methemoglobin Levels

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ABSTRACT

Direct acting methemoglobin forming compounds, such as the well known sodium nitrite, can be added to whole blood leading to *in vitro* formation of methemoglobin. In the canine model the rate of formation of methemoglobin *in vitro* is similar to the rate of methemoglobin formation obtained during rapid intravenous infusion. The *in vitro* addition of direct acting p-aminopropiophenone derivatives to blood obtained from unanesthetized dogs forms methemoglobin at rates similar to the intravenous infusion of the same compounds in the same dogs. These studies demonstrate that *in vitro* addition of compounds to whole blood can be used to rapidly obtain an estimate of the rate of formation of methemoglobin and the approximate whole blood concentration of an unknown compound required to induce desired levels of methemoglobin. Since this *in vitro* system only responds to direct formers of methemoglobin it is possible to determine if a compound which forms methemoglobin in-vivo does so by a direct effect - or if the compound induces methemoglobin through a metabolite.

If a series of methemoglobin formers are to be evaluated the *in vitro* system can be used to identify the most potent and rapid acting of the methemoglobin formers. A comparison of *in vitro* vs. in-vivo activity may identify differences due to altered formation of methemoglobin or to pharmacokinetic differences.

Another *in vitro* system has been developed which is useful in monitoring the rate of interaction between the cyanide ion and methemoglobin. This additional *in vitro* system can be used to determine if the methemoglobin which is formed by one of a

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series of compounds will interact with cyanide at the same rate as methemoglobin which is induced by sodium nitrite.

Introduction: Methemoglobin (MHb) is a derivative of Hemoglobin (Hb), obtained by oxidizing the iron in the heme group (Fig 1).

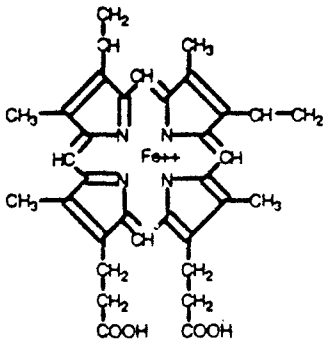

Figure 1

This movement from ferrous (+2) to ferric (+3) gives the molecule a net charge of +1 on each iron and leads to the binding of small anionic ligands such as cyanide (CN-). With the change in oxidation comes a change in color, mHb has a chocolate brown color while Hb, with a high affinity for O2, has a bright red color. This allows for differentiation of the two forms of hemoglobin through spectral analysis.(Lemberg, P. and Legge, J.W. 1949).

Cyanide has been used for decades as a weapon of war, from Napoleon to Hitler, and as recently as Hussein in 1991. The current possible threat of CN- gas in war and industrial accidents warrants the need for a safe, effective CN- antidote. Pedigo first noted the antidotal effects of amyl nitrite against CN- in 1888 without understanding the mechanism of action. (Baskin and Fricke, 1992)

Previous reports have demonstrated that mHb has the capacity to bind CN-. It is this binding property which gives mHb its relevance in these studies. A multitude of compounds induce mHb in various animal species and humans. Therefore, a method which would allow for early detection and quantitation of mHb formation would be extremely valuable. An *in vitro* method would permit preliminary mHb experimentation without exposing an animal to undue drug exposure and eliminating the undesirable time delays inherent to animal studies.

There are a number of mHb-inducing agents which can serve as a source of mHb. Concern exists as to whether the mHb produced in the blood by different drugs is the same. Interest

also lies in the determination of the ratio of interaction between CN- and mHb.

The *in vitro* system is a practical method for assessing direct mHb forming compounds. The %mHb is measured by the Radiometer OSM3 Hemoximeter. This system is capable of measuring samples within one minute, which is necessary due to rapid, continual formation of mHb within the sample. The instrument expresses mHb values as a percent of total Hb found in a volume of blood. The analyses carried out by the OSM3 Hemoximeter are based on spectraphotometric changes.

Methods and Materials: Seven compounds were evaluated in this system: (Fig. 2)

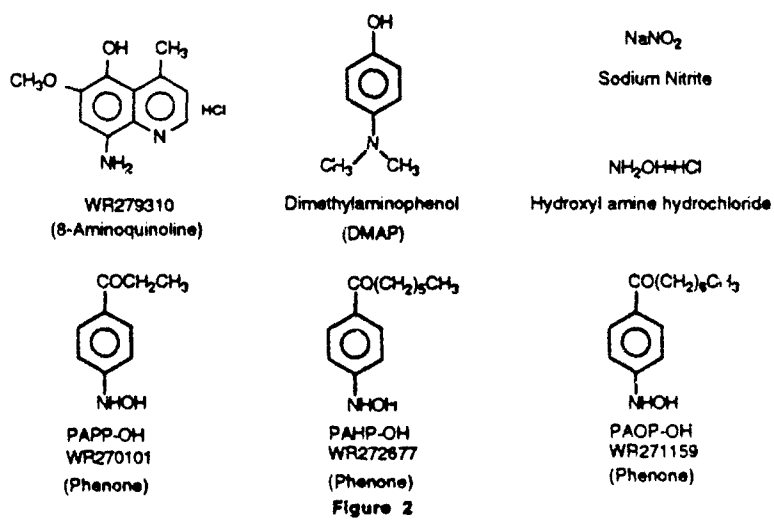

Figure 2

-Hydroxyl amine hydrochloride (HA-HCl) mol. wt= 69.49
-Sodium Nitrite (NaNO2) mol. wt= 69
-Dimethylaminophenol (DMAP) mol. wt= 137
-p-Aminohydroxylpropiophenone (PAPP-OH) mol. wt= 165.2
-p-Aminohydroxyheptanophenone (PAHP-OH) mol. wt= 221.3
-p-Aminohydroxyoctalphenone (PAOP-OH) mol. wt= 235.3
-8-Amino-5-hydroxyl-6-methoxy-4-methylquinoline hydrochloride (WR279,310) mol. wt= 240.6

The compounds were dissolved in PEG-200. Ten ml heparinized blood samples were obtained from each of three unanesthetized animal species: human, dog and sheep. Samples were incubated in a shaker water bath at 37 degrees Celsius for 30 minutes before baseline mHb readings were taken. After baseline sampling, the

test compound solution was added directly to the blood sample with continuing agitation in the water bath for the duration of the experiment. The blood samples were analyzed 30 seconds after mixing and every 1.25 minutes thereafter for 20 minutes and at regular time intervals throughout each experiment on a Radiometer OSM3 Hemoximeter.

***In vivo* Samples:** Unanesthetized Beagle dogs were dosed intravenously with a bolus solution of direct mHb forming compounds. The compounds were dissolved in PEG-200, filter sterilized with a 0.45um 25mm syringe filter. After dosing, samples were drawn from the animal via a catheterized cephalic vein at predetermined time intervals. Samples were then analyzed on a Radiometer OSM3 Hemoximeter within 60 seconds to allow data comparison to these *in vitro* studies.

CN- Formulation Methods: Potassium Cyanide was added to three species of heparinized animal blood to achieve CN- concentrations of 2.0x10-4M to 5.0x10-4M. MHb was induced in 5ml blood samples of the same three species. This sample was then added to a previously CN- spiked 5ml blood sample. Samples were stirred and kept at a constant temperature of 37 degrees C. The rates of CN-/mHb interaction were then determined. Formation and elimination of mHb was analyzed as previously discussed. These data were utilized to determined the ratio of CN-/mHb interaction.

Interaction of mHb with CN-: Data was obtained from the blood of three different species of animal. Potassium Cyanide (Fisher Scientific) was added to the blood of three species (Dog, Sheep, and Human), to develop cyanide concentration of 2.5 X 10-4 M.

Cyanide ion concentration was determined with a cyanide specific ion electrode (Cyanide combination Ion Selective Electrode FK1502CN, combined with an Ion 85 analyzer, Radiometer, Copenhagen). Potential differences were displayed on a strip chart recorder (Kipp and Zonen). A schematic of the system is provided below (Diagram 1).

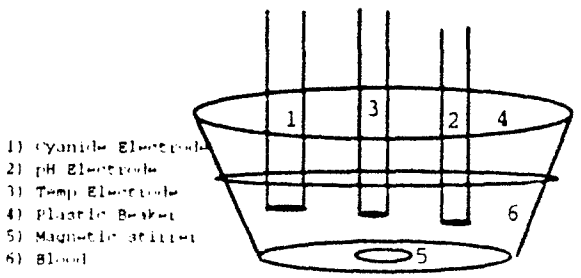

Diagram 1

Methemoglobin was induced by the different methemoglobin-inducing agents. The methemoglobin was then allowed to interact with CN- in 10ml of blood (Dog, Sheep, and Human). The 10mL sample of blood was kept constantly stirring to ensure rapid and complete interaction. Temperature was maintained at 37 degrees Celsius in a Precision Water Bath Model 185 (Precision Scientific Incorporated).

Results: A comparison of *in vivo* to *in vitro* mHb formation (Fig. 3A and B) showed a general similarity in the apparent formation rates from t=0 to Tmax %mHb.

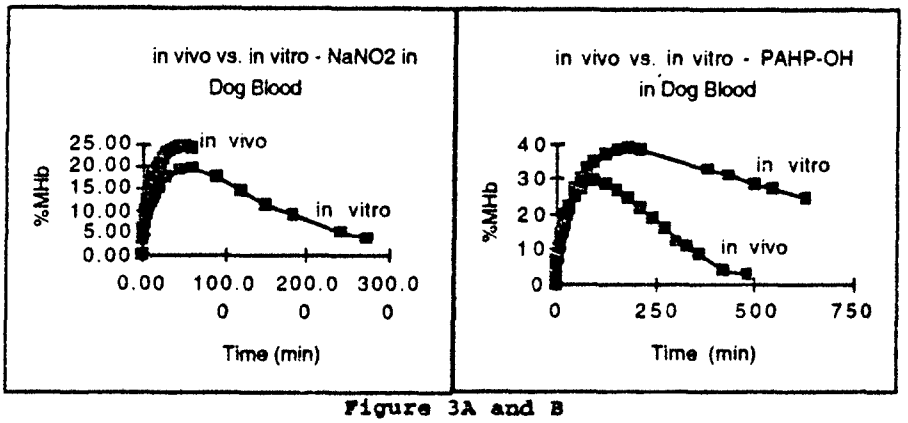

Figure 3A and B

A general relationship was formed between the *in vivo* and *in vitro* data using apparent immediate rates of formation. The apparent rates were determined by plotting the ln(%mHb) against the straight line portion of the graph to determine the apparent rate of formation. Illustrated in Fig. 4A.

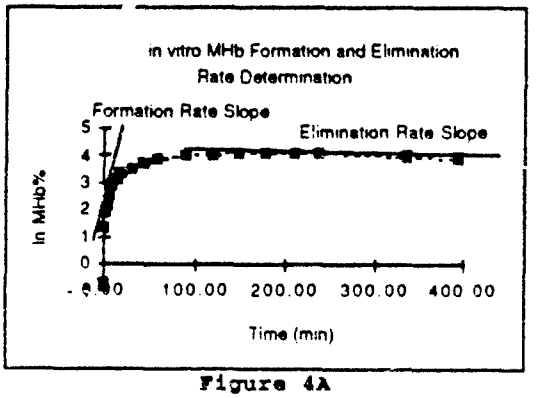

Figure 4A

The *in vivo* and *in vitro* rates of %mHb formation for five compounds were graphed. Using the method described by Fig. 4A, a positive relationship between *in vivo* and *in vitro* formation was demonstrated in Fig. 4B.

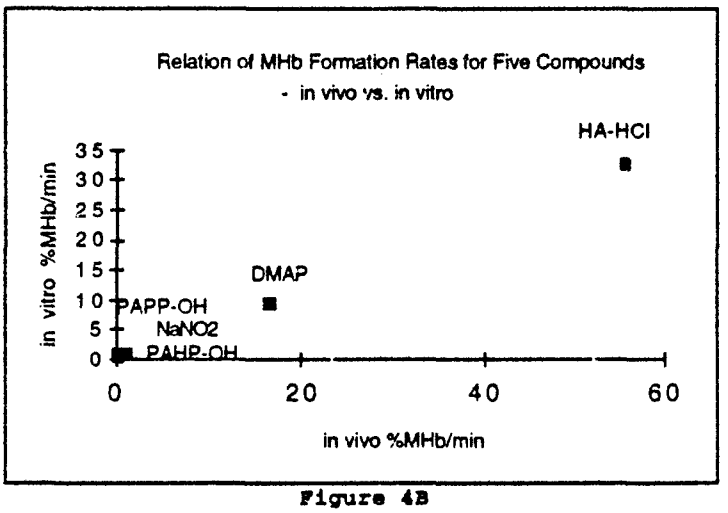

Figure 4B

A comparison of potency was made by finding the extent of mHb formation for each compound in three animal species: dog, human, and sheep blood respectively. Values were found by using maximum mHb% formed when molar equivalents of compounds were added(Fig. 5A, B, and C). From this data, a relationship of potency between the different compounds in different species can be concluded.

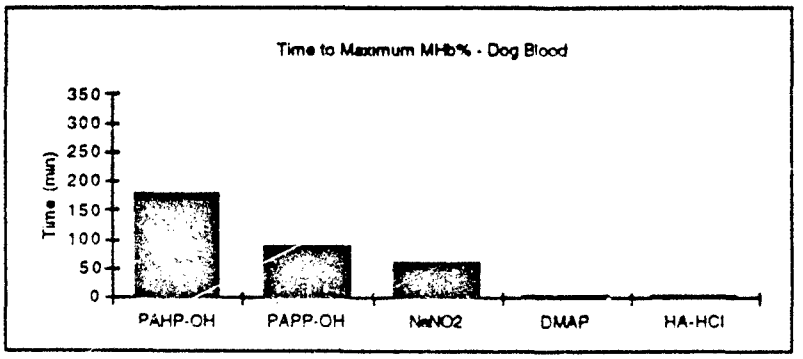

Figure 5A

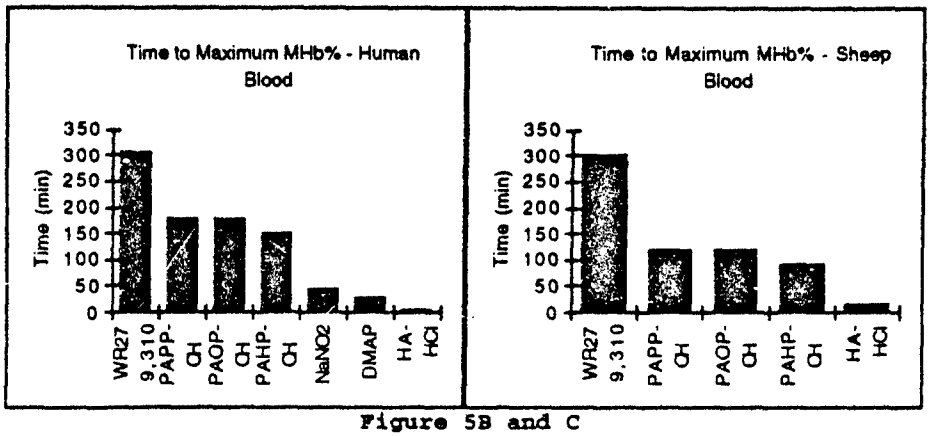

Figure 5B and C

A ranking across species of the time required to attain the maximum (Tmax) %mHb of several mHb forming compounds for three species: dog, human, and sheep respectively was made(Fig. 6A, B, and C). Observed data suggested that interspecies differences exist in both potency and Tmax rankings of mHb forming compounds.

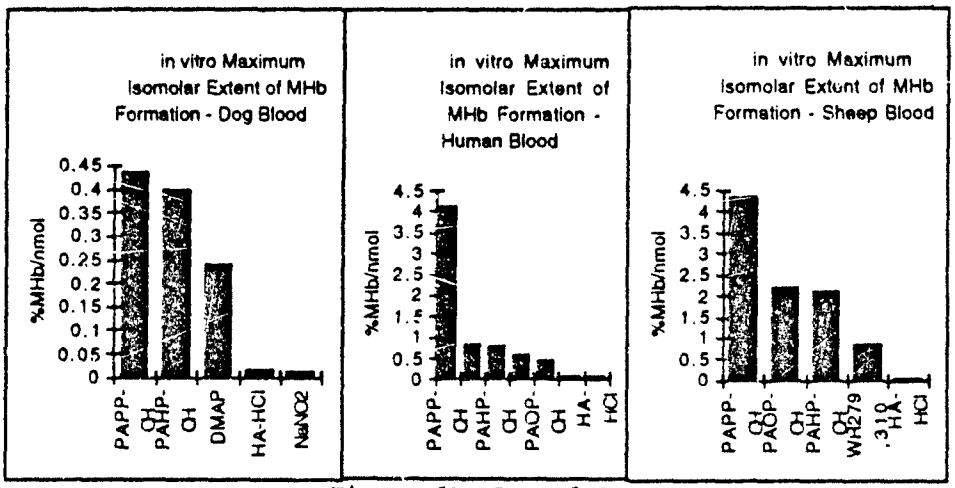

Figure 6A, B. and C

Rate of Interaction between CN- and MHb: The determination of the rate of CN-/mHb interaction in the blood of three species(Sheep, Dog, and Human) was determined in a stepwise procedure (Figures 7, 8, and 9).

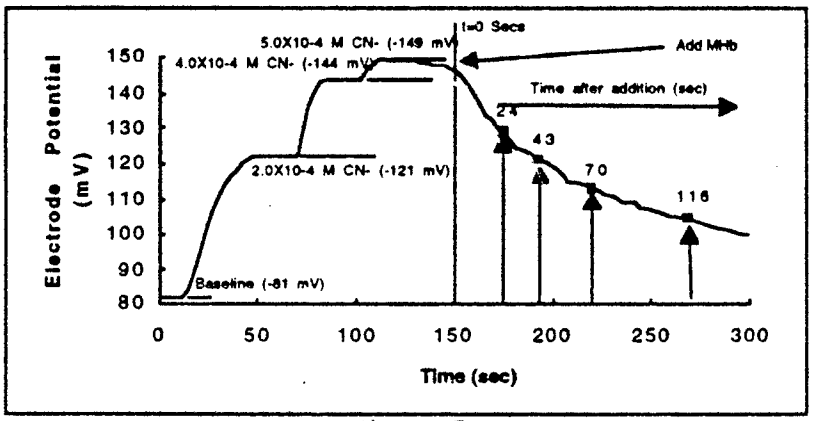

Figure 7

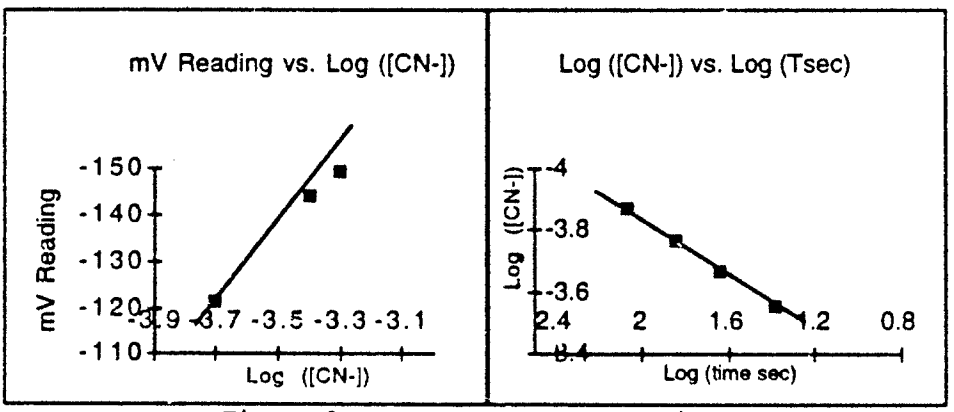

Figure 8 **Figure 9**

Figure 7 illustrates the standard addition of CN- solution and the millivolt response for each standard addition. Also, this figure illustrates the depletion of CN- ion induced by the addition of mHb.

Figure 8 illustrates graphically the standard incremental addition of CN- solution and the millivolt response. In this figure the millivolt response is graphed as a function of the Log molar concentration of CN-.

Figure 9 illustrates graphically the relationship between the depletion of CN-, induced by the addition of mHb, over time measured in seconds.

The rates of interaction between CN- ion and mHb were determined as the slope. The rates were given in moles of CN- per liter per second.

Tables A, B, and C contain the results of the average rates for the interaction of CN- with mHb in the blood of three species.

Average Rate of CN- Interaction with mHb (Sheep)

mHb Inducers	n= number of repeats	Average rate of CN- interaction	STANDARD DEVIATION
DMAP HCl	3	1.90x10-6	2.77x10-7
NaNO2	3	2.59x10-6	5.11x10-7
HA HCl	3	1.48x10-6	6.36x10-7
PAPP-OH	3	4.42x10-6	3.03x10-7
PAOP-OH	3	3.83x10-6	1.42x10-7
PAHP-OH	3	1.64x10-6	3.29x10-7

Average Rate of CN- Interaction with mHb (Dog)

mHb Inducers	n= number of repeats	Average rate of CN- interaction	STANDARD DEVIATION
DMAP HCl	3	1.30x10-6	1.25x10-7
NaNO2	3	1.19x10-6	5.44x10-7
HA HCl	3	2.15x10-6	1.25x10-7
PAPP-OH	3	1.55x10-6	3.98x10-7
PAOP-OH	3	1.15x10-6	4.22x10-7
PAHP-OH	3	1.93x10-6	6.16x10-7

Tables A and B

Average Rate of CN- Interaction with mHb (Human)

mHb Inducers	n= number of repeats	Average rate of CN- interaction	STANDARD DEVIATION
NaNO2	3	1.52x10-6	3.68x10-7
HA HCl	3	1.20x10-6	1.84x10-7

Table C

These results indicate that regardless of the compound used to induce the mHb, once formed mHb will interact with CN- at essentially the same rate and extent.

Ratio of Interaction of CN- to MHb: Table D provides the average ratio of CN- ion interaction with mHb (induced by NaNO2 and HA HCL) in two species (Dog and Sheep).

Average Ratio of CN- Interaction with Methemoglobin

Species	Methemoglobin -inducers	n=number of in vitro repetitions	Average ratio CN-/Methemoglobin
Dog	NaNO2	4	1:1
	HA HCL		2:1
Sheep	NaNO2	4	1:1
	HA HCL		2:1

Table D

Discussion: Once specific direct mHb formers are identified, correlations relating different animal species, different compounds and their apparent rates of Mhb formation

can be derived. When comparing the mHb forming ability of certain compounds, it is important to evaluate the chemical structure and physical properties of these compounds.

A specific compound can be categorized as either a pre treatment or a therapy for CN intoxication based on a rough determination of the apparent mHb formation rate. Compounds for therapy are noted for a rapid formation and are determined by comparing apparent rates of formation against those of known rapid formers such as HA-HCl and DMAP. For therapeutic compounds, how quickly a specific compound can reach therapeutic mHb levels of < 10% is an important factor. The time to maximum mHb (Tmax) is an important consideration for classification of pre treatment compounds. Compounds with greater Tmax's may be used to maintain efficacious levels of mHb for long periods of time, making them useful pre treatment compounds.

MHb that has been induced by different mHb-inducers has been shown to react with CN- at similar rates. Earlier studies conducted with mHb-inducing agents,8-aminoquinolines (e.g., primaquine), have shown that small percentages of mHb (i.e. 5-10%) production can be effective as a CN- antidote (Johnson, 1987). Furthermore, exceptionally rapid mHb formers (HA and DMAP) are able to prevent the lethal effect of CN- following intramuscular injections in doses sufficient to induce 20% mHb (Vick and von Bredow, 1993).

The capability to determine these *in vitro* rates is dependent on optimal operation conditions. The incubation temperature of the water bath was continually monitored and maintained in a range of 35.5 degrees Celsius to 37 degrees Celsius. The pH was also monitored and maintained in the range of 7.35 to 7.5. There were no changes in the pH that were outside this range due to the addition of CN- solution sufficient to establish a concentration of 5 X 10-4 M. During the interaction between CN- and mHb the pH of the solution remained stable as should be expected for decreases in cyanide levels at low concentrations (Lebeda and Deshpande 1990). These conditions were determined by observation to permit reproducible measurements in this system. The optimal range for the measurement of CN- ion with the combination CN- electrode was 10-5 molar to 10-4 molar in this study. Finally, the determination of the ratio for the interaction of CN- with mHb was made possible through the use of the Radiometer OSM3 Hemoximeter.

Although easily performed, *in vitro* studies have some limitations. While this method alleviates some of variables inherent to *in vivo* studies, it adds factors that can not be accurately assessed by quantitative means. Only compounds which can form mHb directly can be evaluated in the *in vitro* model. The *in vitro* system is unable to identify those compounds which are indirect formers.

The similarities of *in vivo* and *in vitro* mHb formation rates noted here are based on very few observations in a single

species. Investigation across species of mHb formation and elimination rate correlations as well as *in vitro* characteristics of potency and Tmax must be further explored to further validate this model. Such work will refine the *in vitro* testing method allowing for more accurate prediction of an unknown compound's effect in an *in vivo* model.

Conclusion:

(1) The *in vitro* mHb formation rate relates to the intravenous formation in the unanesthetized dog model and can be used as an estimate of the mHb formation capability of an unknown compound.

(2) The *in vitro* concentration of a compound required to induce mHb and the time necessary to reach maximal mHb formation varies with the compound tested and the species from which the in vitro blood sample has been obtained.

(3) The rates of interaction between formed mHb and CN- have been demonstrated to be similar regardless of the mHb inducing agent.

(4) The ratio of interaction between CN- and mHb was shown to be 2:1 in the blood of the Dog and the Sheep.

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